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Tumor-Associated Alterations in Caspase-14 Expression in Epithelial Malignancies

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Abstract **Purpose:** Caspase-14 is unique among caspase family proteases in that its proteolytic processing has been principally associated with epithelial cell differentiation rather than apoptosis or inflammation. We investigated caspase-14 expression in several types of human epithelial malignancy by immunohistochemistry, correlating results with stage, histologic grade, and patient survival. **Experimental Design:** Tumor-associated alterations in caspase-14 expression were observed for cervical, ovarian, breast, gastric, and colon cancers. **Results:** In cervical ($n = 445$), ovarian ($n = 91$), and colon ($n = 106$) specimens, expression of caspase-14 was significantly reduced in cancers compared with normal epithelium. Decreases in caspase-14 immunopositivity correlated with the histologic progression of cervical cancer ($P < 0.0001$, ANOVA). In localized gastric cancers, caspase-14 immunostaining was significantly lower in poorly differentiated tumors compared with well-differentiated tumors ($P = 0.02$, Pearson's χ^2 analysis). Lower caspase-14 expression was associated with advanced clinical stage in ovarian cancer ($P = 0.04$, ANOVA) and with shorter overall survival among ovarian cancer patients with serous tumors ($n = 62$) in both univariate ($P = 0.005$) and multivariate ($P = 0.03$) analysis. Lower caspase-14 expression correlated with shorter overall survival among patients with T₃N₀M₀ stage gastric cancers ($n = 94$; $P = 0.006$, log-rank test). In contrast to cervical, ovarian, and colon cancers, caspase-14 expression was increased in ductal carcinoma *in situ* and invasive cancers compared with normal mammary epithelium ($P = 0.001$, t test). **Conclusions:** The findings reveal tumor-specific alterations in caspase-14 expression and suggest that differences in its expression may define subsets of epithelial cancers with distinct clinical behaviors.

Caspases are aspartate-directed cysteine proteases (1), best known for their roles in apoptosis. Many of these proteases cleave and activate other caspases, thus constituting a highly regulated protease network. Eleven caspases have been identified in humans, and they can be divided into three groups based on the domain composition and function (2). Upstream initiator caspases possess long prodomains containing caspase

recruitment domains or death effector domains preceding their catalytic domains and trigger apoptosis or process proinflammatory cytokines. These apoptotic proteases include caspase-2, caspase-8, caspase-9, and caspase-10, whereas inflammatory proteases include caspase-1, caspase-4, and caspase-5 (reviewed in ref. 3). Caspase-3, caspase-6, and caspase-7 constitute a subgroup of downstream effector proteases responsible for apoptosis. These proteins contain short prodomains (3).

Caspase-14 has a very short prodomain (4–6). Based on a phylogenetic analysis, the catalytic domain of caspase-14 is most closely related to the cytokine-activating caspases (2). In contrast to the other short prodomain caspases, caspase-14 fails to undergo proteolytic processing in cultured cells following treatment with apoptosis inducers (5). Also, no substrates of this caspase have been described to date (7). Caspase-14 is highly expressed in embryonic tissues. Expression of caspase-14 mRNA in several adult organs was reported by Ahmad et al. (4), but was claimed to be limited predominantly to epidermis by others (5, 6). Subsequent studies showed transcriptional regulation of caspase-14 expression in simple and complex epithelia (8, 9). Caspase-14 processing was observed during terminal differentiation of epidermal keratinocytes (7, 10), and the determined processing site between Ile¹⁵² and Lys¹⁵³ distinguishes caspase-14 from other caspases that are cleaved at aspartate residues (11). Caspase-14 expression is reportedly

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suppressed in human epidermis when terminal differentiation of keratinocytes is blocked by retinoic acid (12). Regulation of caspase-14 expression by retinoids analogous to proteins of terminal keratinocyte differentiation suggests its involvement in skin barrier formation (12). Also, low expression of caspase-14 in psoriatic skin was elevated by vitamin D3 treatment, enhancing caspase-14 processing in organotypic skin cultures (13). In normal stratum corneum and parakeratotic skin, procaspase-14 is converted to proteolytically processed subunits during terminal keratinocyte differentiation (14).

Recently, peptidolytic activity of recombinant caspase-14 was reported to be induced by granzyme B cleavage when incubated with kosmotropic salts (15), suggesting that the search for potential substrates of caspase-14 should be focused on proteins of the outermost skin layers (14). Thus, caspase-14 is unique among caspase family proteins and may have a different cellular role than related proteins.

The predominantly epithelial expression of caspase-14, and its implicated role in differentiation, evoked our interest in assessing a potential role of this caspase in the malignant transformation of human epithelial cells. This report is the first to describe transformation-associated changes in caspase-14 expression in several types of human malignancies.

Materials and Methods

Patient specimens. Tissue microarrays containing cervical specimens were obtained from the Department of Pathology, Yonsei University (Seoul, Korea). Tissue samples from patients presented between 1998 and 2001 included the cervical intraepithelial neoplasia 1 (CIN1; $n = 47$), CIN2 ($n = 46$), CIN3 ($n = 137$), invasive squamous cell carcinoma ($n = 109$), and normal cervical epithelium adjacent to the transformed cells ($n = 262$). In addition, 26 low-grade and 37 high-grade CIN as well as 43 invasive cervical squamous cell carcinoma specimens were obtained from the Prince of Wales Hospital, Hong Kong. Investigation of specimens for research purposes was approved by the institutional ethics review board in the Chinese University of Hong Kong. Informed consent was obtained from all subjects (16).

A total of 180 nonconsecutive cases of localized gastric cancer were provided by the Department of Pathology at Yonsei University Medical Center. All gastric specimens, derived from patients who presented to the hospital in 2001, were independently reviewed and classified by two pathologists, and areas containing malignant or normal ($n = 99$) gastric epithelium were marked for generation of tissue microarrays. The clinicopathologic parameters, such as tumor-node-metastasis (TNM) stage, tumor differentiation, histomorphologic architecture (according to Lauren's classification), and survival data, were available for statistical analyses. Among the investigated cases, patients with T₂N₀M₀ stage constituted 48% ($n = 86$) of the cohort, whereas 52% ($n = 94$) represented T₃N₀M₀ cancers. The median follow-up period was 7.9 years.

Early-stage colorectal carcinoma specimens were obtained from the Department of Pathology at Yonsei University under institutional review board approval. Tissue samples included 106 nonconsecutive primary tumors derived from patients who presented between 1986 and 1996 with Dukes' B stage [stage II disease, as defined by American Joint Committee on Cancer and Union Internationale Contre le Cancer (AJCC/UICC) criteria]. Patients with Dukes' stage B2 (T₃N₀M₀) constituted 91% of the cohort, whereas 9% represented Dukes' B3 (T₄N₀M₀) cancer. None of these patients received chemotherapy. Clinical data represent a median follow-up of 66 months. Patients with double primary malignancy in other organs and a case of familial adenomatous polyposis coli were excluded from the analysis. Only patients whose primary cause of death was due to

colorectal carcinoma were eligible for inclusion. Individuals whose tumors had positive circumferential margins were not included in the study. We defined right-sided tumors as those originating proximal to the splenic flexure, and left-sided as those arising distal to this site.

Ovarian cancer specimens derived from 91 patients presenting to the Department of Gynecology and Obstetrics of the University of Freiburg/Germany between 1993 and 1998 were included in this study. Clinical data, including survival and chemoresponse data, were available until July 2000, representing a median follow-up of 35 months. This patient cohort was characterized previously elsewhere (17, 18).

Tissue specimens containing normal mammary epithelium ($n = 16$), *in situ* breast carcinomas ($n = 23$), and 121 invasive breast tumors, representing the ductal ($n = 103$), lobular ($n = 15$), and mucinous ($n = 3$) histologic subtypes were obtained from St. Vincent's Hospital in Dublin, Ireland, for tissue microarray generation. The specimens were derived from women who presented in 2001 with symptomatic stage I to III breast cancers, using residual pathologic materials remaining after diagnostic and hormone receptor determination.

Tissue preparation. Normal colon and ovarian tissues for immunohistochemistry analysis were derived from human biopsy and autopsy material obtained under Institutional Review Board approval (Department of Pathology, University of California-San Diego, San Diego, CA). The tissues were prepared for paraffin embedding, as described elsewhere (19).

Tissue microarrays. Tissue microarrays were produced for all investigated tumors and tissues, as described previously (18).

Antibodies. Generation of rabbit polyclonal caspase-14 antiserum (AR-76), using affinity-purified His⁶-tagged recombinant human caspase-14 full-length protein as immunogen, was described previously (20). The monospecificity of the antiserum was confirmed by SDS-PAGE/immunoblot analysis (20). For chicken polyclonal caspase-14 antiserum (ACy-24), similar immunogen preparation and immunization protocols were applied. However, the amount of immunogen per immunization did not exceed 100 µg/500 µL physiologic salt solution and the immunization time was shorter (6 months) compared with that of rabbits. Egg collection started during the second month of immunization and was repeated after every month of boosting for the total period of 6 months. Test bleeds were taken from the wing artery. Termination of the immunization cycle was done by heart puncture exsanguination of anesthetized animals.

Immunohistochemistry. Immunohistochemistry procedure applied to dewaxed tissue sections was described elsewhere (21). Antisera specific for caspase-14 were applied at 1:4,000 (AR-76) and 1:10,000 (v/v; ACy-24). To verify specificity of the results, the immunostaining procedure was done in parallel using preimmune serum or caspase-14 antiserum preabsorbed with 5 to 10 µg/mL of recombinant protein immunogen. The immunostaining scoring system was described previously (18).

For double-labeling experiments, tissue sections were stained using caspase-14 rabbit polyclonal antiserum and mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (DAKO Cytomation, Inc., Burlingame, CA) in conjunction with species-specific secondary antibodies, as described elsewhere (20).

Cell isolation, culture, and generation of cell microarray. Established tumor cell lines were cultured as previously described (22), harvested after fixation, and embedded in paraffin to be arrayed into a tissue microarray block for immunohistochemical screenings. The cell lines included the National Cancer Institute-60 tumor cell line panel (23) plus 14 additional cancer cell lines maintained in our laboratory: seven breast cancer cell lines (231, BT474, HS574, A1N4, 10A, 468, and ZR751), five prostate cancer lines (PPC1, ALVA31, JCA1, LNCaP, and Tsu-PRL), the lymphoma line RS11846, and the endometrium carcinoma line Ishikawa.

Immunoblotting. Human cell lines and specimens derived from normal and malignant human tissues with high ratios of cancer cells relative to stroma (>70%) were selected for immunoblotting analysis.

The protein lysate preparation, immunoblotting procedure, and antigen detection were described previously (24).

Microsatellite instability. Specimens were analyzed for microsatellite instability by PCR amplification of microsatellite markers using DNA from snap-frozen tumor specimens. Extracted DNAs from tumors and matched normal mucosae were PCR amplified at six microsatellite loci to evaluate the microsatellite instability. The markers included the National Cancer Institute–recommended panel of five markers, plus BAT40 (25). PCR products were separated in 6% polyacrylamide gels containing 5.6 mol/L urea, followed by autoradiography. In tumors with microsatellite instability, additional bands were found relative to the normal alleles. Microsatellite instability in three or more markers of which more than two mononucleotide repeat markers were included was classified as MSI-H, whereas all others were classified as microsatellite stable (MSS).

Statistical analysis. Data were analyzed using the STATISTICA software package (StatSoft). The Student's *t* test was applied to characterize protein distribution in normal versus malignant tissues. Differences in the distribution of variables were tested using the Pearson's χ^2 statistics for categorical variables and the ANOVA test for continuous variables. Pearson's correlation coefficients were calculated to assess whether there were relationships between the caspase-14 and PCNA protein levels. To perform survival analysis, the immunostaining data were dichotomized at the median, comparing the clinical outcome for patients whose tumor immunoscores or immunopercents were above the median with those below the median. Overall survival, defined as the time from study entry to death, was determined in univariate survival analysis using the Kaplan-Meier method. Log-rank test was used for correlation of immunostaining data with the patient survival. Multivariate Cox proportional hazards models were fitted to the data to assess which biomarkers were independently associated with overall survival. All factors that had prognostic significance when considered alone ($P < 0.05$) were entered into a multiple regression analysis whereby hazard ratios and significance levels were estimated.

In backward selection, a factor that was not statistically significant was removed from the model until all remaining factors were significant. The 95% confidence intervals for the hazard ratios were calculated by the formula $\exp[\beta \pm 1.960 \text{ SE}(\beta)]$.

Results

Characterization of caspase-14 antibodies and immunoblot analysis of normal human tissues and tumor cell lines. To characterize the specificity of the two polyclonal antibodies, immunoblot analysis was done using recombinant caspase family proteins generated in bacteria and using lysates from human tissues and cell lines (Fig. 1).

As reported by others, procaspase-14 was detected in lysates of human epidermis as a 28 kDa protein, and the respective cleavage products were visualized at 11 and 17 kDa by immunoblot analysis (10, 14). Similarly, analysis of human cancer cell lines detected caspase-14 proform (28–29 kDa) and caspase-14-p17 (8).

Unlike other human caspases, caspase-14 does not undergo catalytic processing under the unusually high concentrations of expression in *Escherichia coli* (15). Thus, in contrast to caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, and caspase-10, which generated large and small subunits of the catalytic domain, recombinant human and mouse caspase-14 was obtained as an unprocessed full-length protein (15). Both rabbit (AR-76) and chicken (AC24y) antibodies raised against purified recombinant human procaspase-14 were determined to be specific for caspase-14, lacking cross-reactivity with other caspase family members (ref. 20) and Fig. 1A, respectively. Reprobing of the blot with His-tag antibody showed loading of

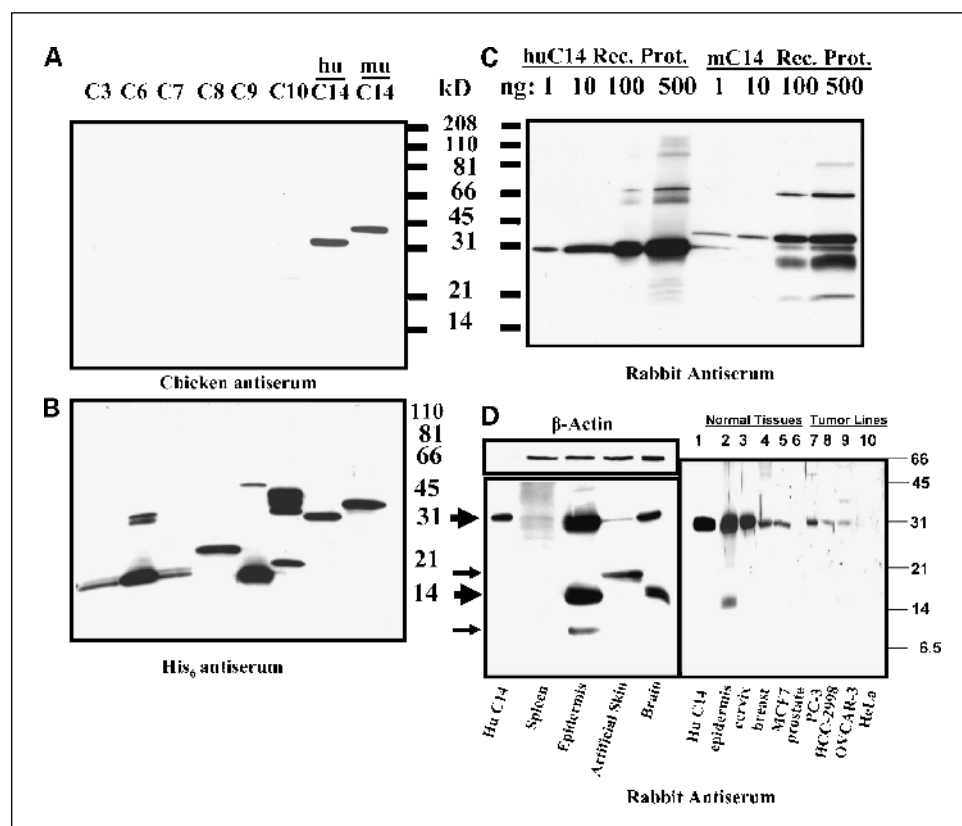


Fig. 1. Characterization of caspase-14 antibodies. A, recombinant purified caspases were subjected to SDS-PAGE/immunoblot analysis, using chicken antibody to caspase-14 (AC-24Y). The difference in gel mobility of human and mouse caspase-14 is consistent with the lengths of these proteins of 242 and 257 amino acids, respectively. B, reprobing of the blot with His-tag antibody showed loading of comparable amounts of all proteases. C, 1 to 500 ng of the recombinant human or murine caspase-14 protein were analyzed by SDS-PAGE/immunoblotting using rabbit antibody to caspase-14 (AR-76). D, lysates from human tissues or cell extracts were normalized for total protein content (50 μ g/lane) and subjected to SDS-PAGE/immunoblot analysis using 1:5,000 (v/v) caspase-14 antiserum (AR-76). Antibody detection was accomplished using an enhanced chemiluminescence method. As a control, 15 ng of recombinant human caspase-14-His⁶ protein was also included in both gels.

comparable amounts of all recombinant proteases (Fig. 1B). Evaluation of different concentrations of recombinant caspase-14 protein showed that as little as 1 ng was easily detected by immunoblotting using both antibodies (Fig. 1C and data not shown).

To further characterize caspase-14 expression, we analyzed lysates from human tissues and extracts from human cell lines by immunoblotting. Both chicken and rabbit antibodies detected caspase-14 proenzyme as a band of ~29 kDa in human epidermis (AR-76 and data not shown; Fig. 1D and E), brain (AR-76; Fig. 1D), cervical and mammary epithelium (AR-76; Fig. 1E), consistent with prior reports showing caspase-14 expression in complex and simple epithelia (8, 15) and in the central nervous system (20). These antisera also reacted with processed forms of caspase-14 in epidermis consistent with prior reports. Interestingly, processed caspase-14 was also detected in brain tissue lysates (AR-76; Fig. 1D). Reprobing the same blot with an antibody recognizing α -actin confirmed loading of equivalent amounts of total protein from all samples.

By immunoblotting, full-length caspase-14 protein was also detected in prostate (PC-3), breast (MCF-7), ovarian (OVCAR-3), and colon (HCC-2998) cancer cell lines but not in a cervical cancer line (HeLa; Fig. 1D). Altogether, these results confirm the specificity of our polyclonal antisera for caspase-14.

Immunohistochemical analysis of caspase-14 expression in human malignancies. Caspase-14 protein expression was investigated in cohorts of patients with cervical, gastric, colorectal, ovarian, and breast cancer. All quantitative data were generated with our rabbit antibody but comparable findings were observed at a qualitative level using the chicken antibody.

Cervical cancer. Tissue microarrays containing cervical specimens derived from Asian women diagnosed with CIN1 (low-grade squamous intraepithelial lesions; mild dysplasia; $n = 47$), CIN2 (high-grade squamous intraepithelial lesion; moderate dysplasia; $n = 46$), CIN3 (high-grade squamous intraepithelial lesion; severe dysplasia-carcinoma *in situ*; $n = 137$), and invasive squamous cell carcinoma ($n = 109$) were stained for caspase-14. Normal cervical epithelium adjacent to the transformed cells was available for each histologic entity ($n = 230$) for all patients in the precancerous groups, and 32 of 109 in women diagnosed with invasive cancer. The highest levels of caspase-14, measured both by immunopercentage and immunoscore (not shown), were observed in the normal epithelium of the exocervix (Fig. 2A and F). In normal cervical squamous epithelium, caspase-14 staining was found mostly in the midzone layer (stratum spongiosum), but was entirely absent from the basal/parabasal cell layer, where mitotically active cells are known to reside, suggesting induction of caspase-14 expression with differentiation. Caspase-14 expression rapidly declined during precancerous stages of malignant transformation, decreasing progressively as the histologic severity of the atypia advanced from CIN1 to CIN3 (Fig. 2B-D, F). The expression of caspase-14 was nearly absent in CIN3 and invasive squamous cell carcinoma (Fig. 2E, F). In contrast to caspase-14, immunoreactivity for cell proliferation marker PCNA was barely detectable in normal cervical epithelium and significantly increased with malignant transformation (Fig. 2F). Thus, decreased expression of caspase-14 correlated inversely with increasing PCNA levels during the neoplastic

process ($r = -0.62$ for comparison of immunopercentage data, $P < 0.0001$).

Our observations were confirmed by immunostaining results obtained on cervical specimens derived from the Prince of Wales Hospital in Hong Kong, showing significantly higher caspase-14 expression in low grade CIN ($n = 26$) compared with high-grade CIN ($n = 37$) and cervical squamous cell carcinoma ($n = 43$; $P = 0.001$).

Gastric cancer. Archival gastric specimens from 180 Asian patients who underwent surgical resection for localized gastric cancer were immunohistochemically analyzed. The primary selection criteria were the availability of formalin-fixed and paraffin-embedded blocks and sufficient clinical follow-up for tumor-specific survival analysis. Using a tissue microarray approach, the expression of caspase-14 was investigated immunohistochemically in T₂N₀M₀ ($n = 86$) and T₃N₀M₀ ($n = 94$) primary gastric carcinomas in relation to patient clinicopathologic features. Caspase-14 was not expressed in the gastric surface epithelium, but positive immunostaining was found in glands deep within the gastric mucosa (not shown). The location of caspase-14 was heterogenous in gastric cancers. When present, the subcellular location of caspase-14 immunostaining in tumors was predominantly cytosolic. However, 38% of diffuse and 10% of intestinal tumors contained both cytosolic and nuclear staining. Ultrastructural analysis recently revealed both cytosolic and nuclear caspase-14 labeling in human epidermis (26). In another study, nuclear translocation of caspase-14 was found to be associated with barrier formation in normal epidermal cells and was markedly reduced in areas of parakeratosis where abnormally high epidermal proliferation occurs (27).

In the investigated cohort, the expression of caspase-14 was significantly associated with histologic architecture and cellular differentiation of adenocarcinomas. As assessed by immunopercentage and immunoscore, caspase-14 was expressed at higher levels in well-differentiated (Fig. 3A) compared with poorly differentiated tumors ($48 \pm 8.3\%$ versus $24 \pm 3.5\%$, $P = 0.004$; $58 \pm 11.1\%$ versus $35 \pm 5.9\%$, $P = 0.02$; Fig. 3B) and in intestinal-type compared with diffuse-type cancers ($P = 0.0001$, $P = 0.006$, respectively; Fig. 3C). Tumors containing signet ring cells contained lower levels of caspase-14 than non-signet ring cell tumors (not shown). Unlike cervical cancers, increased content of caspase-14 correlated positively with elevated PCNA in gastric carcinomas ($r = 0.21$ for comparison of immunopercentages, $P = 0.01$). In contrast, no association was found between caspase-14 protein expression and prominent lymphoid infiltration or mucin content in tumors (not shown).

With respect to clinical outcome, patients with stage T₃N₀M₀ tumors that contained higher levels of caspase-14 (dichotomized at the median) had significantly longer overall survival ($P = 0.04$ for immunopercentage and $P = 0.006$ for immunoscore; Fig. 3D). However, this association with survival was not observed for T₂N₀M₀ stage patients (not shown). In multivariate Cox proportional hazards analysis, caspase-14 remained significant as an independent prognostic factor for the T₃M₀N₀ stage patients (Table 1).

Colon cancer. Tissue microarrays were constructed using primary tumor specimens derived from a cohort of nonconsecutive 106 patients with stage II colorectal carcinoma, who were treated by surgical resection with curative intent. Of the 106 selected cases, 63 patients survived without recurrence,

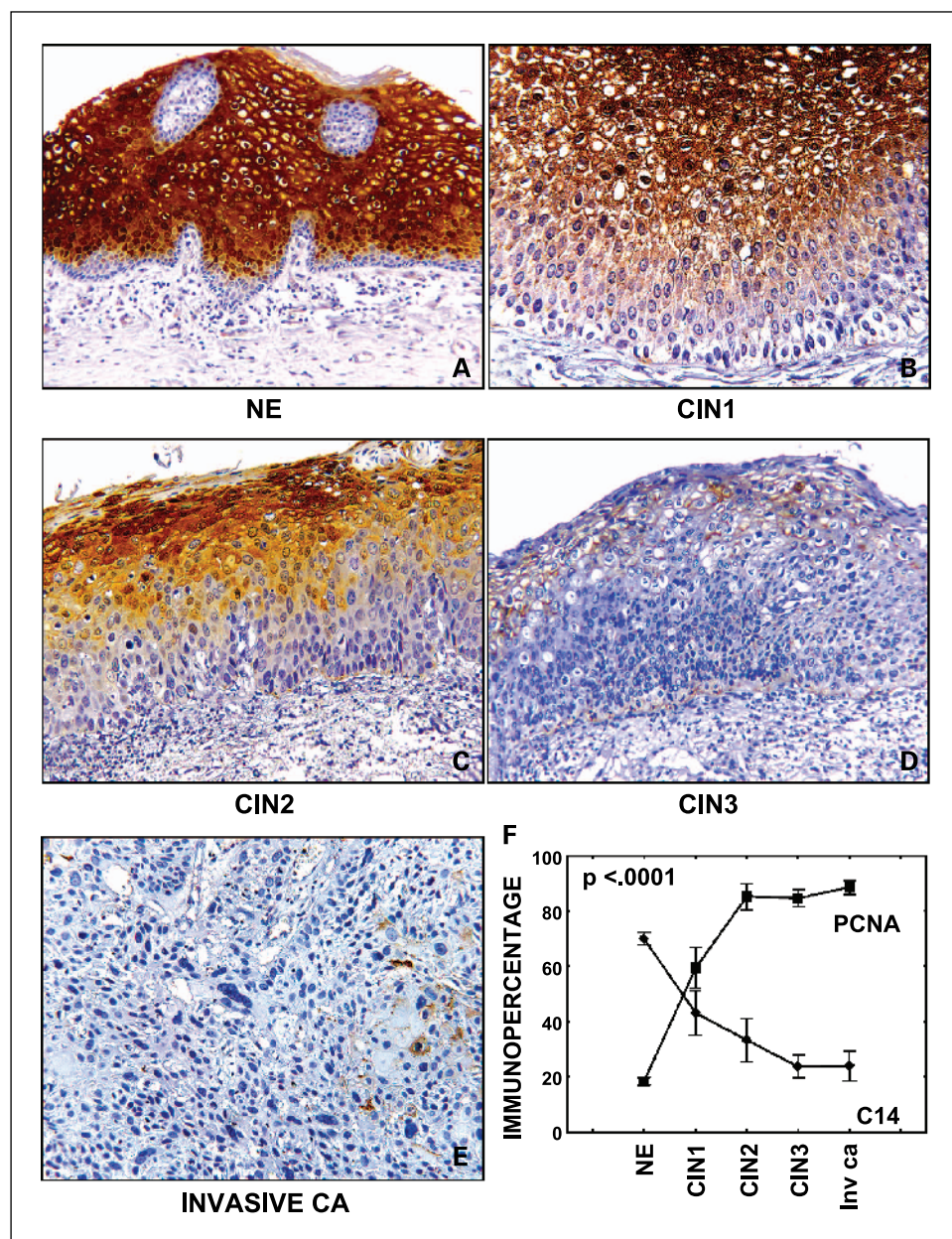


Fig. 2. Immunohistochemical analysis of caspase-14 expression in uterine cervix. Representative caspase-14 immunostaining results are presented showing cervical specimens representing a continuum from normal to malignant epithelium (A, normal cervix; B, CIN1; C, CIN2; D, CIN3; E, invasive carcinoma). Original magnifications, $\times 100$ to $\times 200$. F, graphic presentation of inverse correlations of caspase-14 and PCNA immunostaining during malignant progression in uterine cervix. Means with error plots display the distribution of immunopositivity data for caspase-14 and PCNA with respect to progression of malignant transformation and dedifferentiation of uterine cervix. Points, mean immunopositivity; bars, ± 1.96 SE from the mean.

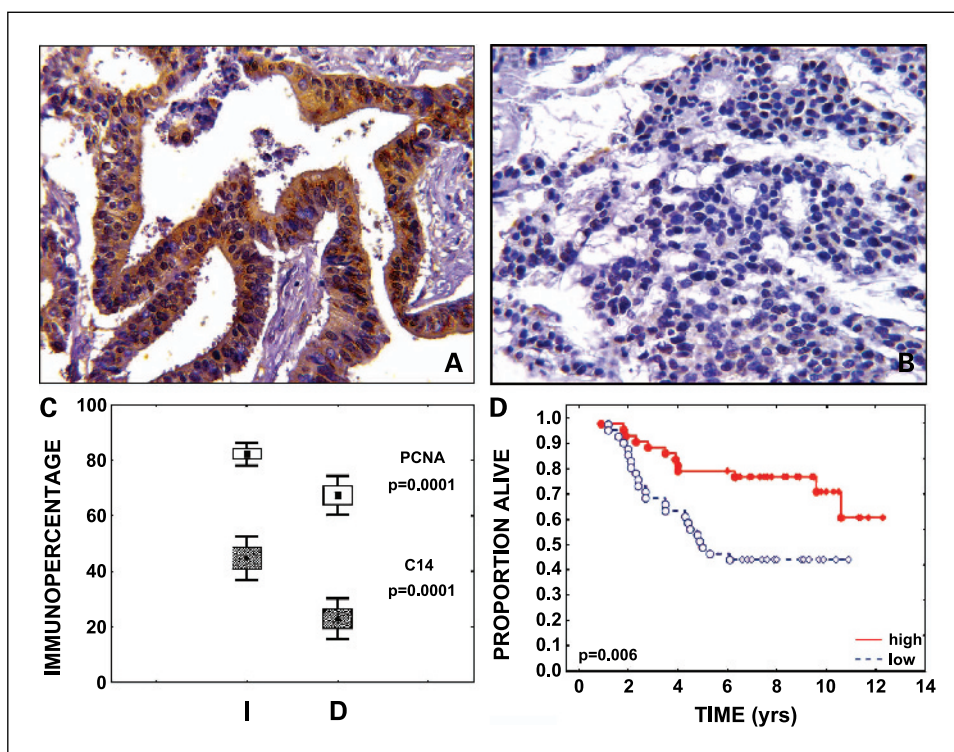
7 patients had recurrent disease, and 36 patients died from colorectal carcinoma. Thus, whereas not an unbiased sequential case series, the survival profile of this cohort closely resembles that of a random population of stage II colorectal carcinoma patients, with 72.5% of individuals alive at 5 years. Adjacent normal colonic mucosa was present in 65% of the 106 tumor specimens on the array, permitting side-by-side comparisons of immunostaining results for normal versus malignant epithelium. In addition, four specimens of normal colon derived from individuals who were not diagnosed with colon cancer were stained separately.

Immunohistochemical analysis of tissue specimens on the microarray chips revealed higher expression of caspase-14 protein, assessed by immunoscore, in normal colonic epithelium compared with the invasive cancer ($P = 0.01$). An opposite tendency was observed for PCNA immunostain-

ing, but statistical analysis did not reveal significant difference between PCNA levels in normal versus malignant epithelium. No correlations were observed in this cohort between overall survival and caspase-14 protein content in tumors. Age, gender, microsatellite instability status, and anatomic location of tumors were not associated with caspase-14 expression.

Ovarian cancer. Caspase-14 expression was investigated using tissue microarrays containing tissue specimens from 91 ovarian carcinoma patients. For six of these patients, specimens of normal ovarian surface epithelium or fallopian tube were also available. The patient cohort comprised 62 individuals with serous carcinomas and 29 cases of nonserous tumors, including mucinous ($n = 13$), endometrioid ($n = 11$), clear cell ($n = 2$), granulosa ($n = 1$), dysgerminoma ($n = 1$), and carcinosarcoma ($n = 1$) types. No significant differences in

Fig. 3. Distribution of caspase-14 and PCNA immunostainings in gastric cancer. Representative caspase-14 immunostaining results are presented for microarrays of gastric specimens in intestinal (A) and diffuse (B) type of gastric adenocarcinoma. C, distribution of immunopositivity data for caspase-14 and PCNA in intestinal versus diffuse cancers; points, means; bars, SE. Caspase-14 immunoscore data were dichotomized into high (red) versus low (blue) expression groups based on the median values. The percentage of patients remaining alive (Y axis) was compounded over time (X axis; in years) by the Kaplan-Meier method (D). The log-rank test was used for correlating the immunostaining data with the patient survival.



caspase-14 protein levels were noted between these two broad histologic categories of ovarian cancer.

When separated into two groups based on stage, the percentage of caspase-14 immunopositivity was significantly higher in low stage carcinomas [Fe'dération Internationale des Gynaecologistes et Obstétristes (FIGO) stage I + II] compared with high-stage tumors (FIGO III and IV; $P = 0.04$; Fig. 4A-C). Caspase-14 immunoscore data did not reach statistical significance ($P = 0.08$), but showed the same trend. In contrast to caspase-14 immunostaining, PCNA immunopositivity was slightly elevated in high-stage compared with low-stage tumors, but the difference was statistically nonsignificant (Fig. 4C). No correlation with caspase-14 expression data was observed for patient age or histologic grade of tumor.

To correlate protein expression with clinical outcome, immunostaining data for the investigated proteins were dichotomized into high versus low expression using median immunopositivity or immunoscore as cutoffs. In univariate analysis, significant correlations were observed in this cohort between longer overall survival and higher caspase-14 immunopositivity ($P = 0.03$) and higher immunoscore ($P = 0.04$) data. In contrast to overall survival, disease-free survival was not significantly different between the two patient groups based on caspase-14 expression. As tested by a Cox's proportional hazards model, high levels of caspase-14 were associated with reduced risk of death from ovarian cancer, with hazard ratio data suggesting a reduced relative risk of ~40% ($P = 0.03$; Table 2). However, in multivariate analysis, only histologic grade maintained independent prognostic significance ($P = 0.002$).

To select a more homogenous cohort of ovarian cancers, we focused on the 62 serous carcinoma cases. The survival advantage associated with higher caspase-14 immunostaining was even more evident in this cohort ($P = 0.006$ for overall

survival; $P = 0.02$ for disease-free survival; Fig. 4D). Moreover, in multivariate analysis of these serous carcinoma patients, the outcome benefit of higher caspase-14 was maintained for overall survival ($P = 0.03$ for caspase-14 immunopositivity; $P = 0.04$ for immunoscore; Table 3 and data not shown).

Breast cancer. Tissue microarrays containing specimens derived from 121 breast cancer patients were immunostained for caspase-14. The tissue samples comprised 23 ductal carcinoma *in situ* tumors, 103 ductal invasive, 15 lobular invasive, and 3 mucinous invasive carcinoma specimens. In addition, 16 normal mammary epithelium specimens, excised from surgical margins, were included on the arrays, as well as four independent samples of normal mammary gland tissue. Unlike other tumors examined in this study, higher caspase-14 immunostaining was found in invasive cancers ($P = 0.0008$; $P = 0.0003$; Fig. 5B) and *in situ* carcinomas ($P = 0.003$, $P = 0.0004$, respectively) compared with normal mammary epithelium (Fig. 5A). Elevation of caspase-14 levels in transformed

Table 1. Multivariate Cox proportional hazards analysis of markers in stage T₃N₀M₀ gastric carcinoma patients

Factors	HR (95% CI)	P
Caspase-14%	0.48 (0.2-0.9)	0.04
Recurrence	26.4 (11.9-58.6)	<0.0001

NOTE: Tumor recurrence, cellular differentiation, histologic architecture, lymphocyte infiltration, mucin content, presence of signet ring cells, and immunopositivity of caspase-14 were included in the univariate analysis. Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

mammary epithelium was accompanied by concomitant gain in PCNA expression ($r = 0.32$, $P < 0.0001$ for immunopositivity; $r = 0.27$, $P = 0.001$ for immunoscore; not shown). In addition to ductal carcinoma *in situ* and invasive cancer, apocrine metaplasia and histologic lesions characterized as apocrine change within sclerosing adenosis contained distinctly increased levels of both caspase-14 and PCNA (data not shown). Thus, elevations in caspase-14 seem to represent a very early alteration in the pathogenesis of breast cancer.

No correlations were found between caspase-14 levels and tumor size, node status, estrogen receptor or progesterone receptor status or patient survival. However, caspase-14 expression was associated with histologic grade of tumors, whereby high-grade tumors contained significantly higher levels of this protein ($84 \pm 2.1\%$ versus $58 \pm 11.1\%$, $P = 0.0009$, for immunopositivity; 148 ± 6.8 versus 91 ± 20.5 , $P = 0.02$, for immunoscore).

Immunohistochemical analysis of caspase-14 protein in the National Cancer Institute panel of tumor cell lines. The relative levels of caspase-14 protein in the National Cancer Institute-60 tumor cell line panel and additional prostate and breast cancer cell lines were characterized by immunohistochemistry analysis using a tissue microarray format. Figure 5C summarizes arbitrary results showing the percentage immunopositivity data for caspase-14. Caspase-14 was detected at a percentage of $\geq 10\%$ in two of seven breast, two of six ovarian, two of three prostate, four of seven colon, one of eight renal, and three of eight lung cancer, two of seven melanoma, two of six glioma, and one of six leukemia cell lines. Automated imaging analysis of caspase-14 immunostaining (Fig. 5D) confirmed these results. Significant levels of

Table 2. Univariate (A) and multivariate (B) Cox proportional hazards analysis among patients with ovarian cancer

Factors	HR (95% CI)	P
A		
Stage	3.5 (1.1-11.3)	0.03
Grade	5.1 (1.8-14.8)	0.002
CA125	3.5 (1.1-10.9)	0.03
Age	1.7 (1.0-2.7)	0.04
Caspase-14%	0.6 (0.3-0.9)	0.03
B		
Grade	5.1 (1.8-14.5)	0.002

NOTE: All factors that showed prognostic significance in the univariate Cox analysis were included in the multivariate Cox proportional hazards model. Only significant results ($P \leq 0.05$) are presented in the table. Grade [low (LMP + G₁) high (G₂ + G₃); stage [low (I + II) high (III + IV)]; age cutoff ≥ 60 years; CA125 cutoff ≥ 50 units/mL.

caspase-14, analyzed by immunoblotting, were previously reported in human epithelial (prostate, breast, stomach, and bladder) cancer cell lines (8).

Discussion

Caspase-14 expression was originally found in simple and complex epithelia (8), and numerous reports linked this protease to keratinocyte senescence (7, 10), suggesting a role

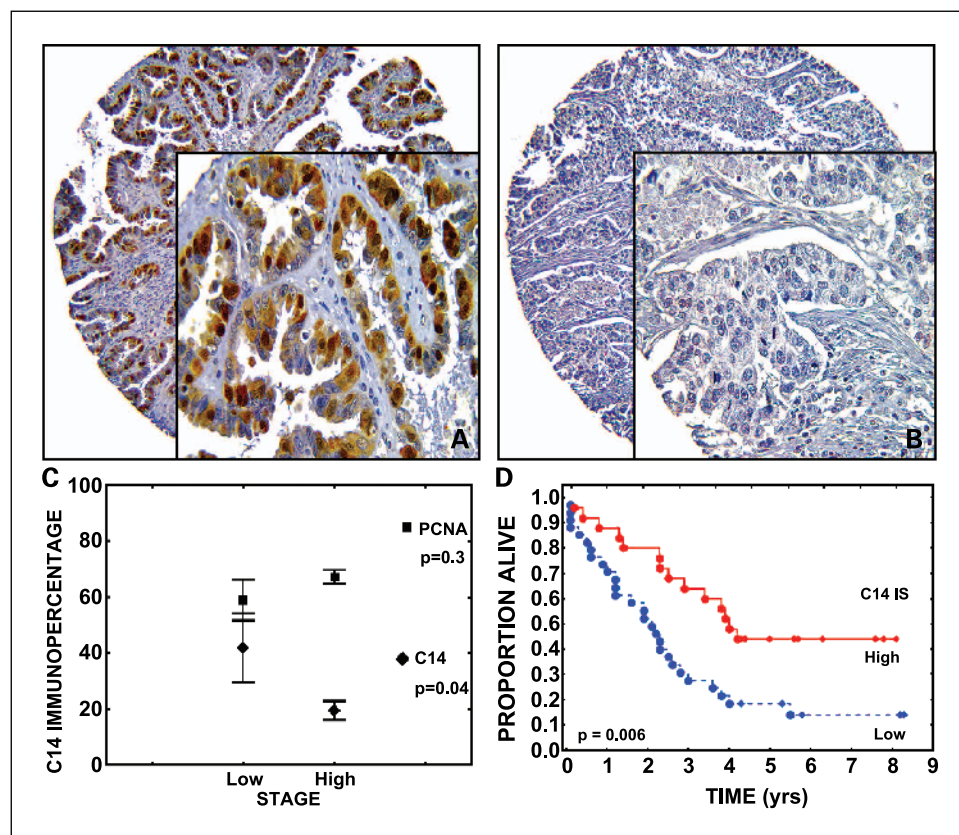


Fig. 4. Immunohistochemical analysis of caspase-14 expression in ovarian cancers. Representative caspase-14 immunostaining results are presented in serous ovarian cancer specimens derived from patients with low (A) or high (B) stage tumors. Original magnifications, $\times 100$ or $\times 400$. C, means and error plots display the distribution of caspase-14 immunopositivity in low-stage (FIGO I + II) compared with high-stage tumors (FIGO III and IV). Points, mean immunopositivity; bars, ± 1.96 SE from the mean. D, the percentage of patients remaining alive (Y axis) was compounded over time (X axis; in years) by the Kaplan-Meier method. The log-rank test was used for correlating the caspase-14 immunoscore data with the survival of serous cancer patients.

Table 3. Univariate (A) and multivariate (B) Cox proportional hazards analysis among patients with serous cancer

Factors	HR (95% CI)	P
A		
Grade	13.3 (1.8-96.2)	0.01
Caspase-14%	0.4 (0.2-0.8)	0.005
B		
Grade	11.2 (1.5-82.0)	0.02
Caspase-14%	0.5 (0.3-0.9)	0.03

for caspase-14 in epithelial cell differentiation. These findings prompted us to investigate caspase-14 protein expression in several types of epithelial malignancies. In this report, we surveyed caspase-14 expression primarily by immunohistochemistry methods in cervical, ovarian, breast, gastric, and

colon cancers. Our findings reveal alterations in caspase-14 expression in association with tumor pathogenesis and progression, with levels of caspase-14 immunostaining decreasing in some malignant diseases (e.g., cervical, ovarian, and colorectal cancers), whereas increasing in others (e.g., breast cancer). The molecular events responsible for these tumor-associated alterations in caspase-14 expression remain to be elucidated.

We observed a progressive loss of caspase-14 expression in cervical cancer progression. Worldwide, cervical cancer is the second most common neoplasm in women, accounting for almost 12% of all female cancers (28). During reproductive years, the endocervical columnar epithelium is transformed into squamous epithelium, delineating the transformation ("T") zone in the region of the original squamocolumnar junction (29). Chronic inflammation also contributes to the development of the T zone, which eventually becomes the main site of origin for premalignant lesions and invasive squamous cell carcinomas of the cervix (29). Cervical cancers are believed to arise through a stepwise progression that correlates with histologic changes constituting a continuum from CINs (grades

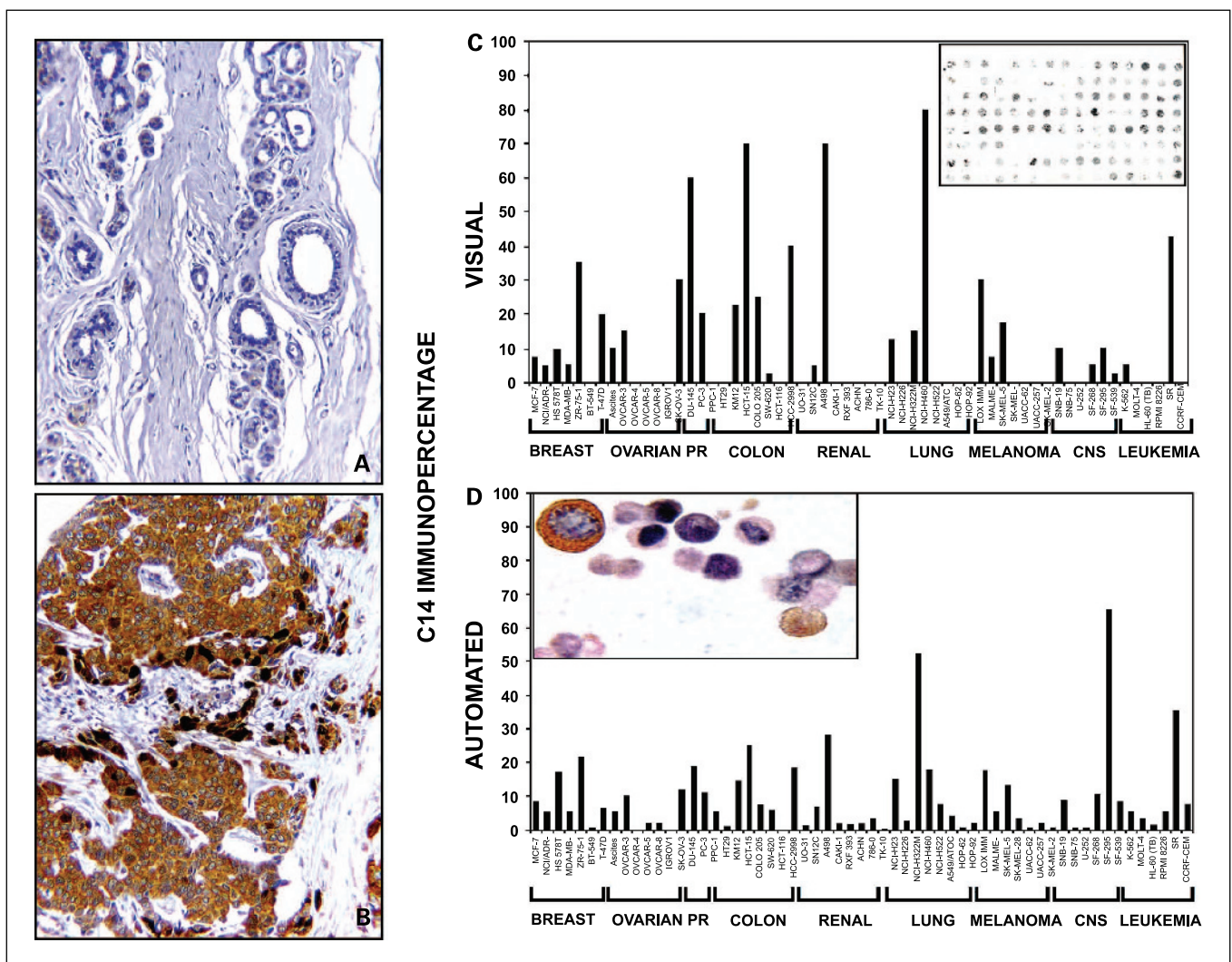


Fig. 5. Distribution of caspase-14 immunostaining in normal mammary epithelium (A), invasive breast cancers (B), and a panel of 60 human cancer cell lines (C-D). A cell line microarray slide was immunostained using AR-76 antibody. The histogram depicts percentage of caspase-14 immunopositivity in particular cell lines, as evaluated by visual inspection (C) or using automated image capture and analysis system Ariol SL-50/MB-8TM (Applied Imaging, San Jose, CA; D).

1-3) to squamous cell carcinoma or adenocarcinoma of the cervix (30).

Declining levels of caspase-14 observed during dedifferentiation of cervical epithelium in the process of malignant transformation parallel a reported function of this protein during terminal maturation of epidermal keratinocytes (7, 10). Recently, differential expression of caspase-14 in UV-induced mouse skin squamous cell carcinomas compared with normal skin was identified by serial analysis of gene expression profiling (31). The subsequent Northern blot analysis revealed 8.5-fold decrease of caspase-14 in squamous cell carcinomas (31). Thus, our investigation uncovered a similar tendency in human cancer derived from transformed cervical squamous epithelium. Also in our study, PCNA, which is expressed in cells that have entered the cell cycle, was dramatically increased with progressing transformation in preneoplastic cervical lesions, inversely correlating with caspase-14 expression. Thus, withdrawal from the cell cycle and commitment to terminal differentiation may be linked to pathways controlling caspase-14 expression. Interestingly, a possible involvement of activated caspases in terminal differentiation has been documented for a variety of normal cell types, such as rodent lens epithelial cells, maturing sperm cells, osteoblasts, and monocytes (32–35).

Gastric cancer is the second most frequent cause of death from cancer in the world today. The Lauren's classification recognizes two distinct histologic types (i.e., intestinal and diffuse; ref. 36), with the latter having a worse prognosis. In our investigation of localized stomach cancers, caspase-14 was associated with well-differentiated histology and the intestinal architecture, such that caspase-14 staining was present at significantly higher levels in well-differentiated intestinal tumors compared with poorly differentiated diffuse cancers. Caspase-14 immunostaining also conferred independent prognostic value for patients with T₃N₀M₀ stage disease, with retention of caspase-14 expression associated with better survival. Thus, caspase-14 may provide a useful marker for delineating differences in the clinical behavior of gastric cancers, and therefore warrants further investigation with respect to its potential prognostic utility.

The pathogenesis of intestinal-type gastric carcinomas is believed to be promoted by chronic inflammation. In this regard, gastric infection with *Helicobacter pylori* is causally associated with chronic gastritis, atrophic gastritis, and intestinal metaplasia. In this regard, based on a phylogenetic analysis, the catalytic domain of caspase-14 is most closely akin to caspase-1, caspase-4, and caspase-5 (2), which take part in the intracellular activation of the proinflammatory cytokines interleukin-1 β and interleukin-18 (reviewed in ref. 37). Considering the role of chronic inflammation in gastric cancer

pathogenesis, it is plausible that caspase-14 plays a role in the generation of protective immunity against the bacterial pathogens and chronic inflammation through effects on cytokine production. Similar arguments could be made for cervical cancer, which is often associated with chronic viral infection with human papilloma virus. It remains to be established, however, if caspase-14 proteolytically activates proinflammatory cytokines.

In colorectal carcinoma patient cohort, caspase-14 levels were significantly higher ($P = 0.01$) in normal colonic epithelium compared with stage II adenocarcinoma. Unlike gastric cancer, no associations were uncovered with clinicopathologic characteristics in the patient cohort we examined.

Normal ovarian epithelial cells contain abundant caspase-14 staining whereas cancers tend to lose expression of this protease. We found an association between caspase-14 expression and low-stage disease (FIGO I and II), suggesting that loss of caspase-14 expression is associated with progression of ovarian cancer. However, longitudinal studies are required to determine whether loss of caspase-14 expression is indicative of transformation of ovarian cancers to more aggressive tumors.

In contrast to cervical, ovarian, gastric, and colorectal cancers, elevated caspase-14 expression was found in invasive breast cancers and ductal carcinoma *in situ* compared with normal mammary epithelium ($P = 0.01$). The inverse correlation between caspase-14 expression and differentiation seen in neoplastic mammary gland tissue shows that caspase-14 is not a global marker of cell differentiation. Although not formally addressed here, it is also unlikely that caspase-14 expression is a general marker of growth arrest based on the findings obtained for mammary gland lesions and gastric cancers, as well as our finding of highly diverse expression of caspase-14 in cultured cancer cell lines. Delineating the mechanisms responsible for altered caspase-14 expression in cancers may eventually provide insights into the tissue-specific pathogenic mechanisms responsible for carcinogenesis and uncover specific signaling pathways that govern the production of this protease. Elucidating the biochemical and cellular functions of caspase-14 may also provide new avenues for understanding tumor biology and devising therapeutic strategies.

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